FINAL REPORT ON A SHORT-TERM

GRANT RESEARCH PROJECT

304/PPSP/6131133

KALLIKREIN-KININ SYSTEM IN HUMAN FETOPLACENTAL TISSUES IN PREGNANCY-INDUCED HYPERTENSION

DR. MAHANEEM BINTI MOHAMED

Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di Pusat Pengajian.

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1. Pembentangan Poster di "16th Scientific Meeting of The Malaysian Society of Pharmacology and Physiology" pada 25-26 June 2001 di International Medical University,K.Lumpur. Bertajuk " Kininogen in Fetoplacental Tissues from Normotensive Pregnant Women and Women with Pregnancy-induced Hypertension".
2. Pembentangan Oral di "7th National Conference on Medical Sciences" pada 17-18 May 2002 di Universiti Sains Malaysia, Kelantan, bertajuk " Levels of kininogen and tissue kallikrein activity in Fetoplacental Tissues from Women with Pregnancy-induced Hypertension".
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Macintosh computer. Renal nerves were isolated and placed on bipolar electrodes and its activity measured using the above system. Saline (0.9% w/v NaCl) was infused at 3 mL/h and the experiment began 2h later. Following the collection of baseline data, bolus doses of rilmenidine, 100, 300 and 1000 mg/kg were administered and the saline infusion was increased, such that the animal received 0.25% body weight/min over 40 min, and blocks of recording were made every 5 min for volume expansion treatment and changes in MAP, HR and RSNA were recorded. The data obtained was compared using 2 way ANOVA and was considered to be significant ($p<0.05$).

The results obtained demonstrated that at all doses of rilmenidine there were decreases in the SBP in both groups of animals. A similar situation was observed for DBP at all doses in the SHRs but only at doses above 300 mg/kg in the DSHRs. HR was decreased at doses of more than 300 mg/kg in DSHRs, but in SHRs only at 1000 mg/kg. In SHRs, rilmenidine caused significant reduction in RSNA at doses 300 mg/kg and 1000 mg/kg. However, in DSHRs rilmenidine caused significant reduction in RSNA only at a high dose (1000 mg/kg). During acute volume expansion, there were no meaningful changes in the SBP and HR in both groups of animals, but the DBP and RSNA were suppressed. Futhermore, there were significant overall differences in DBP and RSNA between SHRs and DSHRs.

In summary, the sympathoinhibitory action of rilmenidine and volume expansion appeared to be reduced in diabetic spontaneously hypertensive rats suggesting that in diabetes, the central drive generating renal sympathetic nerve activity was resistant to regulation by neural pathways involving putative imadazoline receptor activation.

KININOGEN IN TERM FETOPLACENTAL TISSUES FROM NORMOTENSIVE PREGNANT WOMEN AND WOMEN WITH PREGNANCY-INDUCED HYPERTENSION.

M Mahaneem, ET Larmie, HJ Singh. Department of Physiology, School of Medical Sciences, Universiti Sains Malaysia, Kelantan, Malaysia.

The pathogenesis of pregnancy-induced hypertension (PIH) remains an enigma. It is postulated that imbalance of placental vasoconstrictor and vasodilator substances may contribute to the placental hypoperfusion in PIH[1]. Therefore the objective of this study was to estimate the levels of kininogen, a precursor of kinin (a potent vasodilator[2]), in fetoplacental homogenates obtained from normotensive pregnant women and women with PIH.

A portion of chorion laeve, amniotic membrane, placental plate chorion, fetal placenta (fetal surface of placenta) and maternal placenta (surface of placenta attached to the uterine wall) were obtained from 8 normotensive pregnant women and 8 women with pregnancy-induced hypertension after receiving informed consent. Each tissue was washed thoroughly with Krebs-Hensleit(KH) buffer, pH 7.4, dried between filter papers, weighed and frozen in buffer solution at –20°C until when needed. Tissue homogenate was prepared in relevant volume of KH buffer using a homogenizer. After centrifugation, the supernatant was dialyzed overnight at 4°C against Tris-HCl buffer, pH 7.4. Kininogen was further trypsinized to generate kinin. The released kinin was then estimated by EIA. Kininogen values were expressed as pg bradykinin equivalent/mg of tissue. All results were presented as mean SEM. Statistical analysis was performed using a non-parametric test, Kruskal-Wallis test, and a p value of <0.05 was considered significant.

Kininogen levels were significantly ($p<0.05$) lower in chorion laeve, amniotic membrane, placental plate chorion, fetal placenta and maternal placenta (114.26±44.95, 267.39±109.23, 251.82±52.14, 156.60±21.37, 122.22±25.71 pg bradykinin Eq/mg tissue respectively) from women with PIH compared to the corresponding tissues from normotensive pregnant women. Kininogen levels were found to be significantly lower in the fetoplacental tissues obtained from women with PIH. This may reflect a decreased kininogen production and hence a reduction in kinin formation. This alteration may therefore play a role in placental hypoperfusion seen in PIH.

1. Walsh SW. AM. J. Obstet. Gynecol. 1985; 152: 335–340.
2. Bhoola KD, Figueroa CD and Worthy K. Pharmacol. Rev. 1992; 44: 1–80.

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Effects of Valsartan, an angiotensin II antagonist, on glucose induced insulin secretion

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Introduction
Diabetes is a known risk factor in coronary heart disease. A number of antihypertensive drugs are known to be diabetogenic. This may contribute to the less than expected decrease in the incident of coronary heart disease with reduction in blood pressure with treatment in hypertensive patients.

Objective
This study is to determine the effects of a member, Valsartan, of a new class of drugs, angiotensin II antagonists, on glucose induced insulin secretion.

Methodology
Male albino rat pancreases were used. The isolated pancreases were perfused with Kreb's solution containing bovine albumin (200mg/dl) with low glucose (60mg/dl) followed by high glucose (300mg/dl) at a rate of 4 mls/mins. The dose of Valsartan is based on the peak blood level achieved in human at standard single dose of 1.64 mg/L. Five treatment groups were used: Control, Valsartan at 10%, 100% and at 10 times therapeutic level, and with Diazoxide 10 ug/ml which is known to inhibit approximately 50% of insulin secretion. Insulin levels in the perfusate were measured by radioimmunoassay.

Results
Valsartan at particularly lower doses, significantly increase the glucose induced insulin secretion ($P<0.05$). Valsartan at 10 % therapeutic level, at therapeutic level and at 10 times therapeutic level, increases glucose induced insulin secretion by 180%, 134% and 129% respectively. Diazoxide as expected, significantly inhibit glucose induced insulin secretion ($P < 0.05$).

Conclusion
Valsartan stimulates glucose induced insulin secretion particularly at lower therapeutic levels.

Levels of kininogen and tissue kallikrein activity in fetoplacental tissues from women with pregnancy-induced hypertension

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Introduction
The pathogenesis of pregnancy-induced hypertension (PIH) remains an enigma. It is postulated that an imbalance of vasoactive substances may contribute to the placental hypoperfusion in PIH.

Objective
To estimate the levels of kininogen and tissue kallikrein activity, the substrate and enzyme that responsible to generate kinin (a potent vasodilator), in fetoplacental tissues obtained from women with normal pregnancy and PIH.

Methodology
A portion of amnion, chorion leave, placental plate chorion, fetal placenta and maternal placenta were obtained from 12 women with normal pregnancy and 12 women with PIH. Each tissue was washed throughly with Krebs-Henseleit buffer, blotted dry, weighed and frozen until used. Level of kininogen and both active and total tissue kallikrein activity were estimated in each tissue.

Results and Conclusion
Kininogen levels were found to be significantly lower in chorion laeve, placental plate chorion, fetal placenta and maternal placenta in women with PIH. No significant differences were found for active, total and calculated inactive tissue kallikrein activity in various fetoplacental tissues for both groups. However, the overall kininogen/ tissue kallikrein ratio, which reflects the overall kinin production in whole fetoplacental unit, was found to be significantly lower in women with PIH. In addition, there was a negative correlation between this ratio to maternal diastolic blood presuure. These findings may therefore confirm the reported preponderance of vasoconstrictor substances that might account for placental hypoperfusion in PIH.

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ABSTRACT

The pathogenesis of pregnancy-induced hypertension (PIH) remains an enigma. However, it is currently postulated that placental hypoperfusion due to an imbalance of vasoconstrictor and vasodilator substances in the placenta may contribute to the pathogenesis of PIH. Hence, the objectives of this study are: (1) to determine the kininogen (using enzyme immunoassay) and tissue kallikrein levels (using a synthetic chromogenic substrate, S-2266), the substrate and enzyme, respectively, that are responsible for the generation of kinin (a vasodilator); (2) to determine the molecular weight of tissue kallikrein (by Western blot analysis); and (3) to determine the gross effect of fetoplacental tissue extracts on the contraction of rabbit jejunum smooth muscle using various fetoplacental tissues from 12 normotensive pregnant women and 12 women with PIH. No significant differences were found for active, total and inactive tissue kallikrein levels in tissues from both groups. However, kininogen levels and kininogen/total tissue kallikrein ratios were significantly lower in chorion laeve, placental plate chorion, fetal placenta and maternal placenta from women with PIH. The molecular weight of tissue kallikrein was about 52 kDa for both groups. These findings might indicate a lower level of kinin generation in fetoplacental tissues of women with PIH confirming the reported preponderance of vasoconstrictor activity. The higher increase in smooth muscle tone produced by application of the fetoplacental tissue extracts from women with PIH also further supports this postulate.

SISTEM KALLIKREIN-KININ DI DALAM TISU FETOPLASENTA YANG DIPEROLEHI DARIPADA WANITA YANG MENGHIDAP HIPERTENSI AKIBAT KEHAMILAN (ABSTRAK)

Patogenesis hipertensi akibat kehamilan (HAK) masih belum diketahui. Walau bagaimanapun, kini ia dipostulat bahawa hipoperfusi plasenta akibat ketidakseimbangan bahan vasokonstriktor dan vasodilator mungkin menyumbang kepada patogenesis HAK. Oleh itu, objektif kajian adalah: (1)menentukan aras kininogen (menggunakan imunoesei enzim) dan tisu kallikrein (menggunakan substrat kromogenik sintetik, S-2266), substrat dan enzim, masing-masing, yang bertanggungjawab menghasilkan kinin (satu vasodilator); (2)menentukan berat molekul tisu kallikrein (menggunakan analisis blot Western); dan (3)menentukan secara kasar kesan ekstrak tisu fetoplasenta ke atas kontraksi otot licin jejunum arnab dengan menggunakan beberapa tisu fetoplasenta daripada 12 wanita hamil yang normal dan 12 wanita yang menghidap HAK. Tiada perbezaan yang signifikan didapati bagi aras tisu kallikrein aktif, total dan inaktif di dalam tisu dari kedua-dua kumpulan. Walaubagaimanapun, aras kininogen dan nisbah kininogen/tisu kallikrein total adalah lebih rendah secara signifikan di dalam korion laeve, korion plat plasenta, fetal plasenta dan maternal plasenta dari wanita yang menghidap HAK. Berat molekul tisu kallikrein untuk kedua-dua kumpulan adalah lebih kurang 52 kDa. Penemuan ini mencadangkan penghasilan kinin yang lebih rendah di dalam tisu fetoplasenta wanita yang menghidap HAK selaras dengan laporan mengenai aktiviti vasokonstriktor yang lebih tinggi. Peningkatan tonus otot licin yang dihasilkan melalui aplikasi ekstrak tisu fetoplasenta daripada wanita yang mengidap HAK menguatkan lagi sokongan terhadap postulat tersebut.

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2. OBJECTIVES

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1. INTRODUCTION

Hypertensive disorders of pregnancy have been reported to complicate almost 10% of all pregnancies (Gifford *et.al*, 1990). Among these disorders is pregnancy-induced hypertension (PIH), which refers to both gestational hypertension and preeclampsia (Davey & MacGillivary, 1988). PIH not only predisposes mothers to severe complications such as abruptio placenta, cerebrovascular accident, end-organ failure, disseminated intravascular coagulation and death but also predisposes fetuses to intrauterine growth retardation, intrauterine death and iatrogenic preterm delivery (Gifford *et.al*, 1990).

Despite the serious impact of PIH on maternal and fetal health, research into its pathogenesis still remains an enigma. It is presently believed that the placenta plays a central role in the pathogenesis of PIH. This is evidenced by the fact that delivery of the placenta or termination of the pregnancy seems to be the only definitive cure for this condition. The clinical manifestations have been attributed to a reduced uteroplacental perfusion (Trudinger *et.al*, 1985). It is presumed that the ischaemic placenta then releases a stress factor(s) into the maternal circulation that cause(s) a generalized maternal endothelial dysfunction resulting in multiple pathophysiological changes and the subsequent clinical manifestations of PIH (Baker *et.al*, 1995 & Brockelsby *et.al*, 2000).

Defective placentation has been postulated to cause placental hypoperfusion in PIH (Sheppard & Bonnar, 1981 & Khong et.al, 1986). A number of postulates

have been proposed to explain the possible cause(s) for the defective placentation. These include: a genetic predispositon (Cooper *et.al*, 1988 & Lachmeijer *et.al*, 1998), an immune maladaptation (Campbell *et.al*, 1985 & Colbern *et.al*, 1994) and an intrinsic defect of the trophoblastic function (Zhou *et.al*, 1997).

Another plausible cause proposed for the placental hypoperfusion is an imbalance of vasodilator and vasoconstrictor substances. This possibility is attributed to the fact that placenta has no neural innervation (Reilly & Russell, 1977 & Khong *et.al*, 1997), hence, it is plausible that regulation of placental circulation may be due mainly to the action of inherent vasoactive substances. Thus, a higher level of vasoconstrictor substances over vasodilator substances within the placenta may cause vasospasm and subsequent placental hypoperfusion. This possibility is borne on one hand by the fact that the levels of vasoconstrictors such as endothelin-1 (Singh *et.al*, 2001) and thromboxane (Walsh, 1985) have been reported to be higher in placentas obtained from women with PIH as compared to those in normal pregnancy. On the other hand, the levels of vasodilator such as prostacyclin (Walsh, 1985) and nitric oxide synthase, enzyme for the production of another vasodilator, nitric oxide (Brennecke *et.al*, 1997), have also been reported to be lower. It is also possible that an imbalance of vasoactive substances within the placenta during early stages of gestation may precipitate placental hypoxia that may also result in defective placentation (Genbacev *et.al*, 1996). However, to date, no data is available on the level of placental kinin (another potent vasodilator) or its

components kininogen and tissue kallikrein that form the kallikrein-kinin system (KKS) in placental tissues of women with PIH.

The presence of kininogen (Hermann *et.al*, 1996), tissue kallikrein (Valdes *et.al*, 2001a) and kinin B_2 receptors (Valdes *et.al*, 2001b) have been reported in normal human fetoplacental tissues. This indicates that kinin is produced locally in the placenta and therefore might also play a role in the regulation of the placental blood flow. Kinin has a very short half-life for it to be measured directly, therefore, in this study the level of kinin is indirectly estimated by the determination of the levels of kininogen and tissue kallikrein, the substrate and enzyme respectively, that are responsible for the production of kinin in tissues.

2. OBJECTIVES

1. To determine the levels of kininogen in various fetoplacental tissues obtained from normotensive pregnant women and women with PIH.
2. To determine the levels of active, total and inactive tissue kallikrein in various fetoplacental tissues obtained from both groups.
3. To determine the molecular weight of tissue kallikrein in the various fetoplacental tissues obtained from both group since this has hitherto not been determined and to see any differences between the groups.
4. To determine the gross effect of fetoplacental extracts, obtained from both groups, on smooth muscle tone to test the hypothesis of an imbalance of vasoactive substances in the placenta.

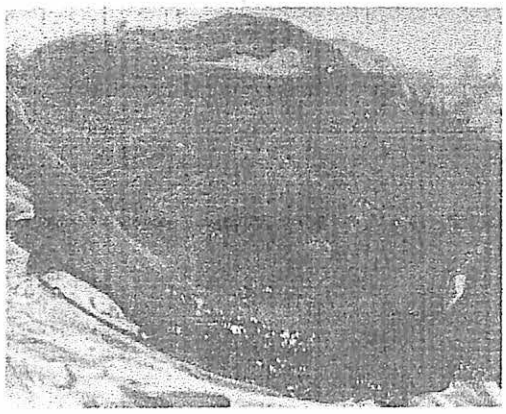
3. METHODS AND MATERIALS

3.1 Subject selection

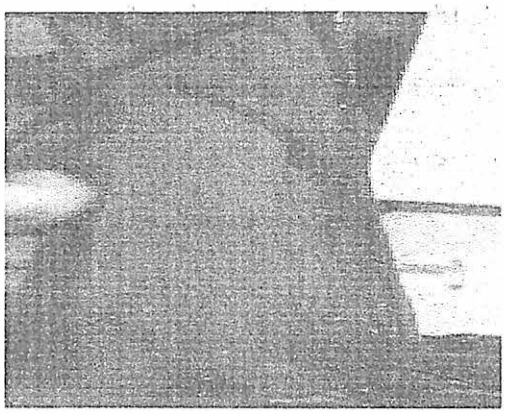
After receiving an informed consent, fresh placentas were obtained from 12 normotensive pregnant (NP) women and 12 women with pregnancy-induced hypertension (PIH). For women with PIH, the inclusion criteria included: a diastolic blood pressure of ≥ 110mmHg on one occasion or ≥ 90mmHg on two occasions, taken at least four hours apart, that developed after 20 weeks of gestation with the presence or absence of proteinuria, and a return of the blood pressure and abnormal urine to normal values within 6 weeks after delivery (Davey & MacGillivray, 1988 & Brown *et.al*, 2001). The exclusion criteria included: age above 40 years, delivery before third trimester of pregnancy, presence of hypertension or proteinuria before pregnancy or before 20 weeks of gestation, presence of underlying diabetes mellitus, renal disease or hepatitis and multiple pregnancies. Controls consisted of NP women with no medical illnesses. All subjects were matched for race, maternal age and gestational age. Data consisting of maternal age, gestational age at sampling, systolic blood pressure (SBP) and diastolic blood pressure (DBP) before delivery and neonatal birth weight were recorded.

3.2 Preparation of fetoplacental tissues

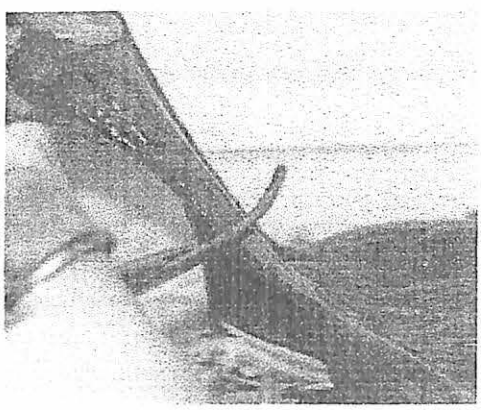
Portions of amnion, chorion laeve, placental plate chorion, fetal placenta (fetal surface of placenta) and maternal placenta (surface of placenta attached to the uterus) were dissected from each placenta as described by Singh *et.al* (2001) (Figure 3.1). Each tissue was then placed in a plastic test tube containing Krebs-Henseleit (KH) buffer (Appendix A). It was immediately transported to the



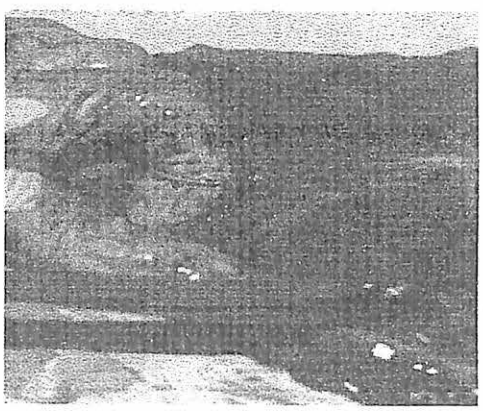
Placenta and its membranes



Amnion



Chorion laeve



Placental plate chorion

Fetal placenta

Maternal placenta

Figure 3.1: Photographs showing dissected portions of amnion, chorion laeve, placental plate chorion, fetal placenta and maternal placenta from a placenta and its membranes.

laboratory where each of the tissue was put on separate plastic petri dishes and washed thoroughly by renewing the buffer several times to remove the blood. Subsequently the tissues were dried between filter papers, weighed and frozen in KH buffer at –80°C for analysis at a later date (Singh *et.al*, 2001 & Sharma *et.al*, 1998).

3.3 Preparation of samples from various fetoplacental tissues for determination of kininogen and tissue kallikrein levels

Preparation of samples for kininogen and tissue kallikrein levels determination was done as described by Sharma *et.al* (1998). Each tissue was separately homogenized in the relevant volume of KH buffer (200mg tissue/ml buffer) with a homogenizer (IKA Labortechnique, Germany). After centrifugation at 3000rpm for 10 minutes at 4°C, the supernatant was dialyzed overnight at 4°C against 0.01M Tris-HCl buffer, pH 7.4 (Appendix B) in a beaker, that contained a magnetic bar and was placed on a magnetic stirrer. The dialyzed supernatant was used as a sample for further assays.

3.4 Determination of kininogen level in each sample

Kininogen level in each sample was determined by trypsinizing the sample to generate kinin as in 3.4.1. The released kinin was estimated by enzyme immunoassay (competitive binding technique) using Markit-M Bradykinin kit (Dainippon Pharmaceutical Co., Ltd, Japan) (Sharma *et.al*, 1998) (Figure 3.2). The released kinin in the sample and the peroxidase-labeled bradykinin were allowed to react competitively with rabbit anti-bradykinin antibody, which was captured by goat anti-rabbit IgG antibody coated on each microstrip well. The

kinin concentration was determined from the enzyme activity of peroxidase-labeled bradykinin bound to anti-bradykinin antibody. The kininogen value was expressed as bradykinin equivalent/mg of tissue. The specificity of Markit-M Bradykinin to bradykinin and lys-bradykinin (kallidin) was 100%.

3.4.1 Preparation of a pre-treated sample

Preparation of a pre-treated sample was done as follows:

1. Each sample was diluted in deionized water (1:5 dilution).
2. 1ml of diluted sample in a test tube was boiled in a water bath for 10 minutes (100°C). This was allowed to cool and then incubated with 20μg trypsin, TPCK (Sigma, USA) for 60 minutes at 37°C, in the presence of 3mM 1-10 phenanthroline (Sigma, USA; Appendix C).
3. The reaction was stopped by adding 100μg of soybean trypsin inhibitor (SBTI; Sigma, USA) for 20 minutes and then boiled in a water bath for 2 minutes.
4. Then 0.5ml of sample was treated with 0.1ml 20% trichloroacetic acid (a deproteinizing agent; from the kit), mixed and centrifuged at 3000rpm for 10 minutes at 4°C.
5. 0.25ml of supernatant was added to 0.25ml Buffer B (from the kit) and ready as a pre-treated sample.

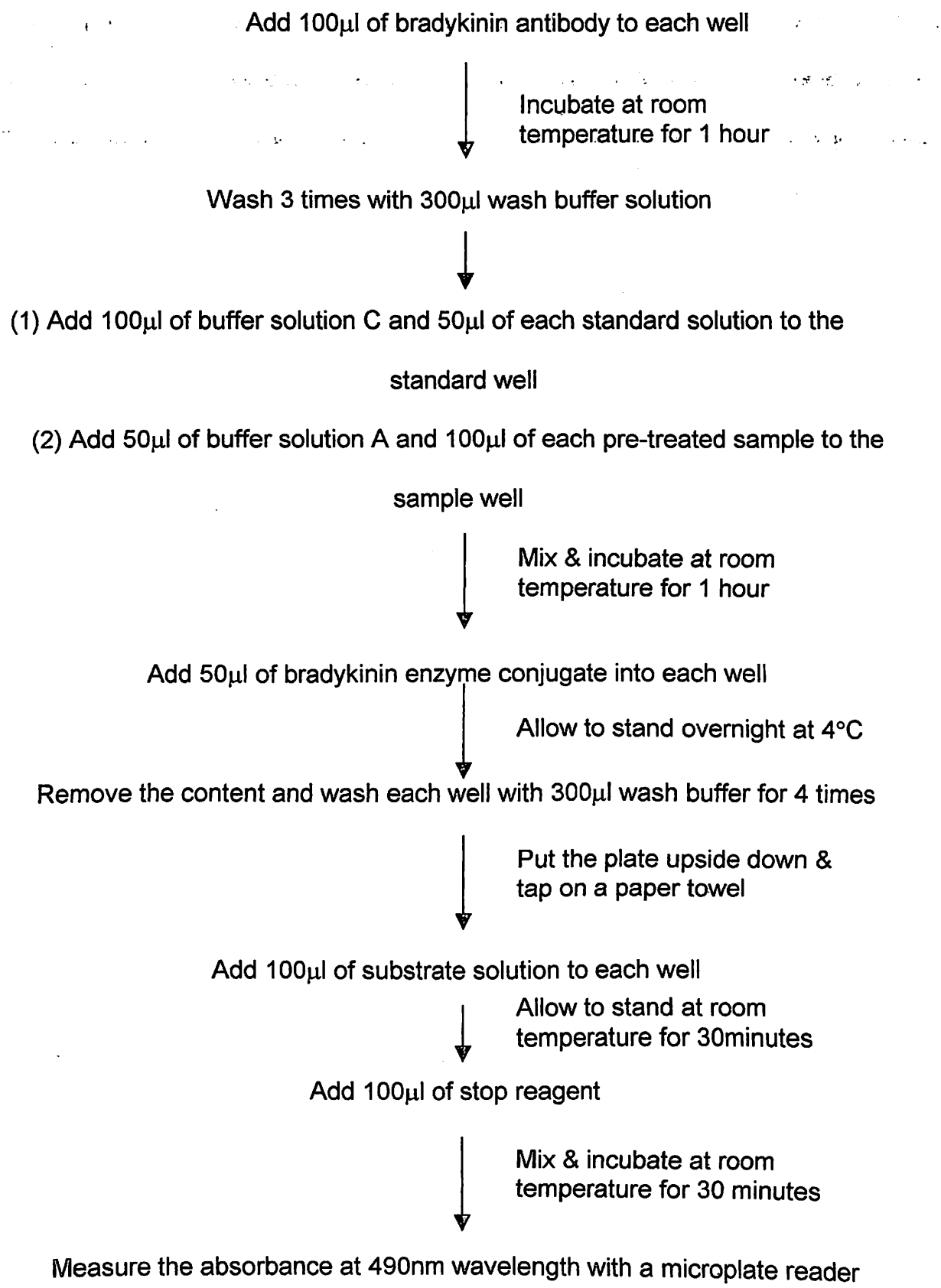


Figure 3.2: Flow chart showing assay procedure to determine the kininogen level using Markit-M Bradykinin kit.

3.5 Determination of tissue kallikrein level in each sample

Tissue kallikrein level was determined by the activity of tissue kallikrein on a synthetic chromogenic substrate, H-D-Val-Leu-Arg-pNa (S-2266; Chromogenix Instrumentation Laboratory SpA, Italy), which was specific for tissue kallikrein. Tissue kallikrein hydrolysed this substrate and the rate of paranitroaniline (pNA) formation, which was proportional to the tissue kallikrein level in each sample, was measured using a spectrophotometer (JASCO, Japan) (Sharma *et.al,* 1998).

The time interval during which the rate of paranitroaniline formation increases linearly with the increasing concentration of tissue kallikrein for the purified tissue kallikrein activity on the substrate was determined. The upper limit of this time interval was taken as an optimum time for tissue kallikrein activity. This optimum time was then used to prepare a standard graph for tissue kallikrein. The enzyme level in each sample was obtained from this standard graph and then calculated as kallikrein unit in 1mg tissue (ku/mg). One kallikrein unit (ku) hydrolyzed 1.0μmol of S-2266 to paranitroaniline (pNA) per minute (Sharma *et.al*, 1998).

3.5.1 Determination of the optimum time for purified tissue kallikrein activity

The determination of the optimum time for purified tissue kallikrein activity was done as follows using 60ku/ml of porcine pancreatic tissue kallikrein (Sigma, USA) (Appendix D):

1. 2250µl of 0.05M, pH 9.0 Tris HCl (Appendix E) was incubated at 37°C in a sterile plastic tube.
2. After 5 minutes, 50µl of 60ku/ml purified tissue kallikrein was added, mixed well and incubated for 2 minutes at 37°C.
3. Then 200µl of 2mM S-2266 was added (Appendix F) and mixed well for it to be hydrolyzed by tissue kallikrein. (Blank solution was also prepared by using the same reagents as above in the same order but substituting 200µl of Tris HCl buffer for the substrate S-2266 solution)
4. The absorbances for purified tissue kallikrein activity at 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 minutes were obtained using a spectrophotometer at a wavelength of 405nm.
5. The results obtained are the mean values of triple determinations. A graph of the absorbance of 60ku/ml purified tissue kallikrein activity (A) against time (minute) was plotted (Figure 3.3).
6. Using the linear portion of this graph, another three graphs of the absorbance of 60ku/ml purified tissue kallikrein against the times of 0 to 3 minutes, 0 to 4 minutes and 0 to 5 minutes were plotted (Figure 3.4, 3.5 & 3.6, respectively). Among these graphs, the graph obtained for 0 to 4 minutes was found to be the most linear graph with the highest correlation ($r = 0.996$ and $A/min = 0.12$; Figure 3.5) (Sharma *et.al*, 1998).
7. Hence, four minutes was chosen as the optimum time for tissue kallikrein activity (i.e. the rate of paranitroaniline formation increases linearly with the increasing concentration of kallikrein up to 4 minutes). It was then used to prepare the standard graph for tissue kallikrein level and also to determine the tissue kallikrein level in each sample.

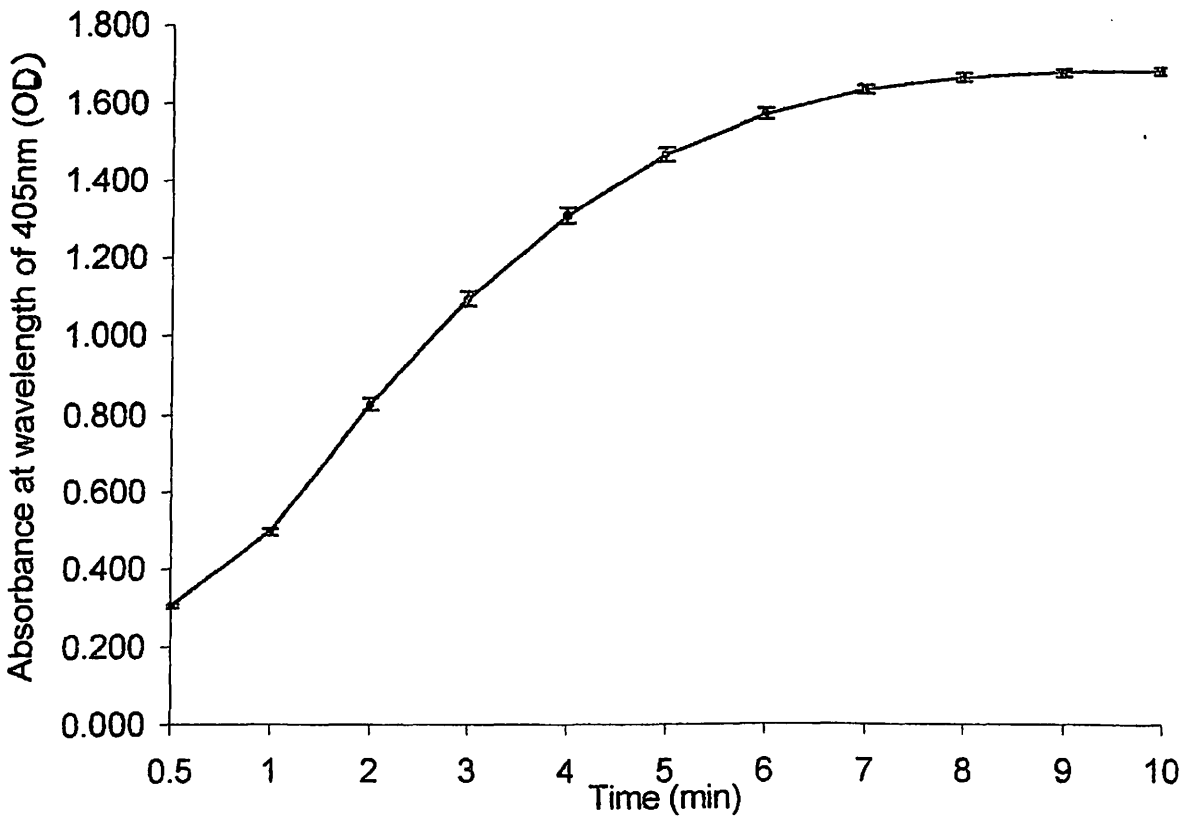


Figure 3.3: The absorbance of 60ku/ml purified tissue kallikrein activity against time.

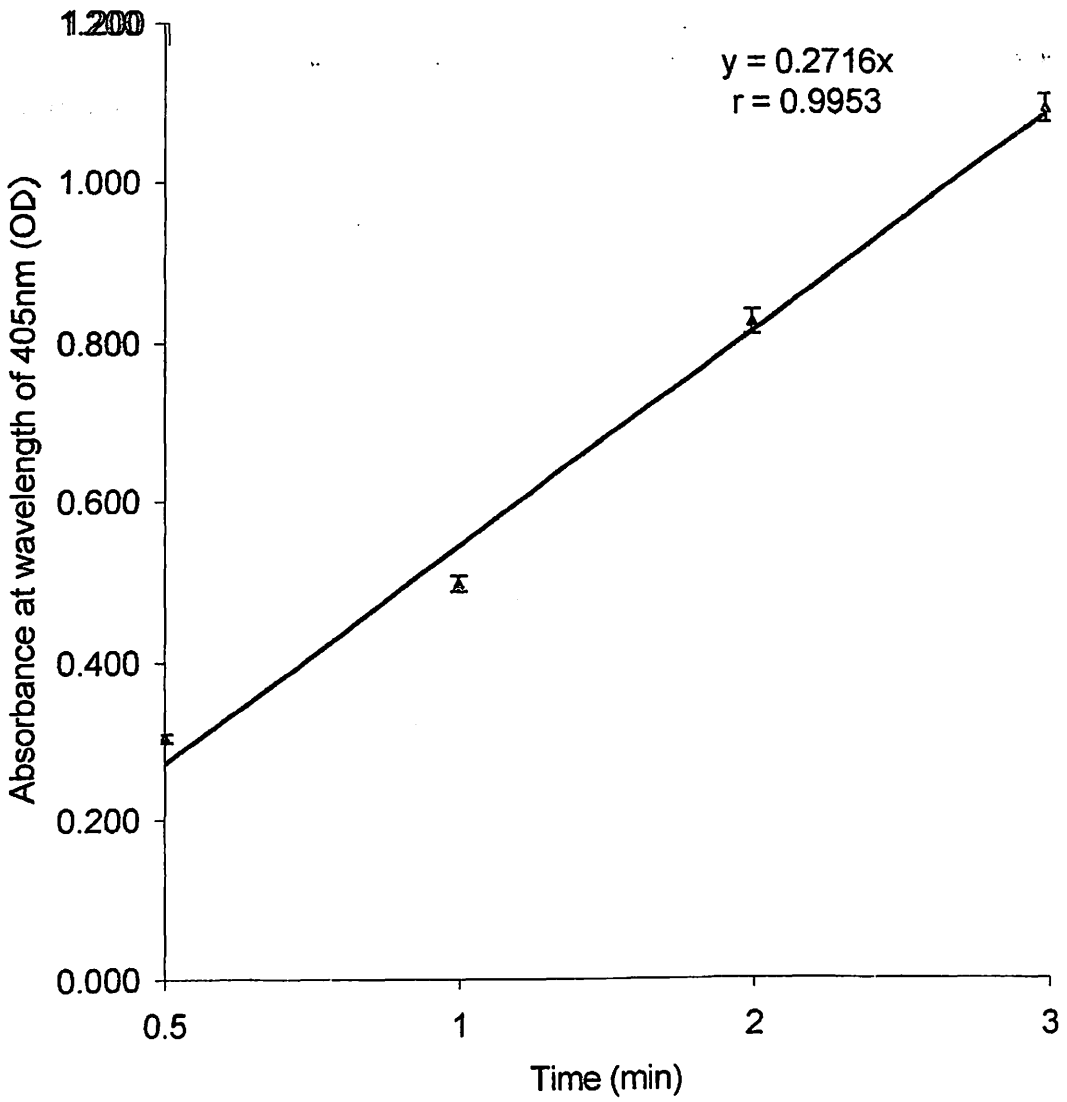


Figure 3.4: The absorbance of 60ku/ml purified tissue kallikrein activity against the time of 0 to 3 minutes.

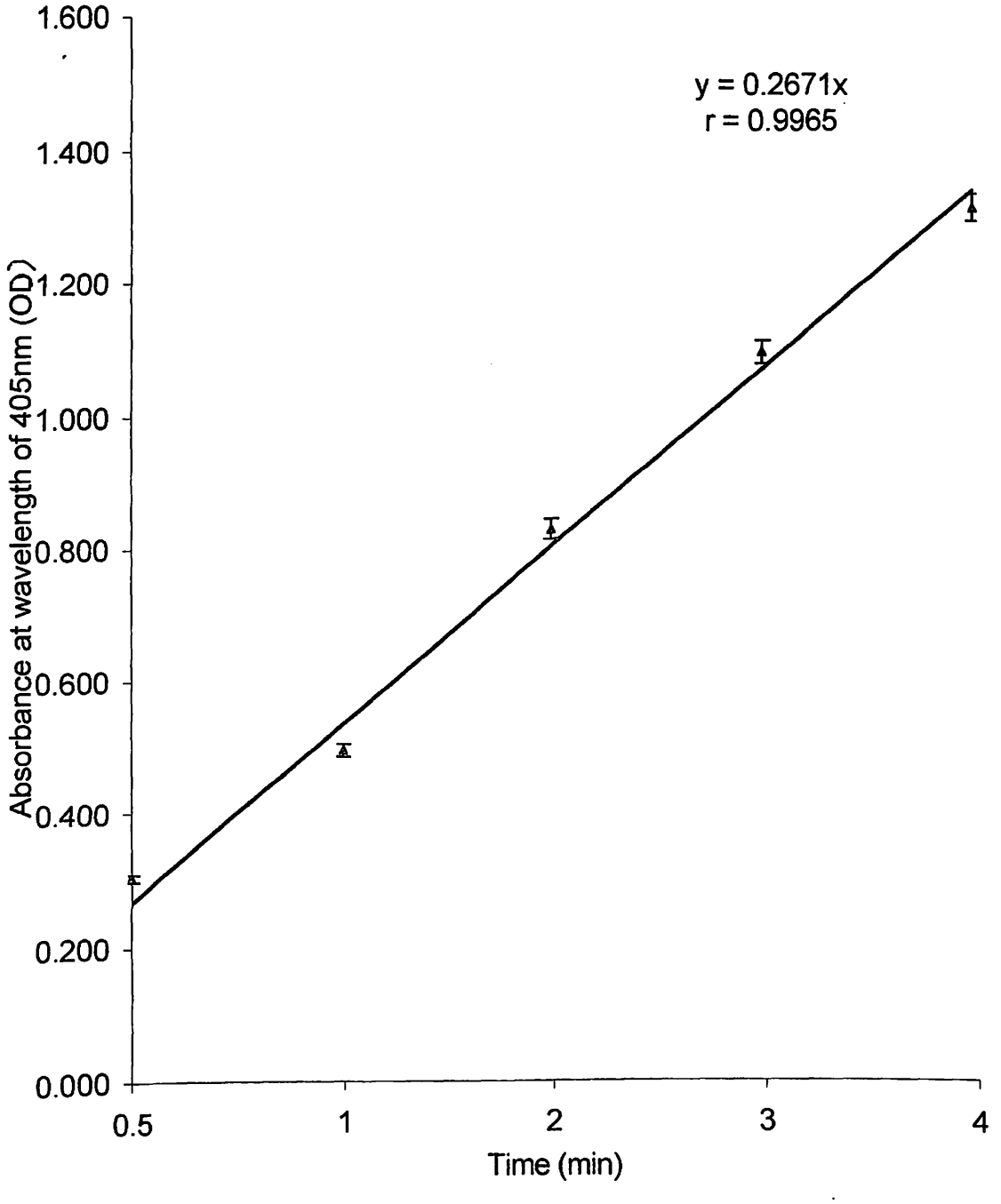


Figure 3.5: The absorbance of 60ku/ml purified tissue kallikrein activity against the time of 0 to 4 minutes.

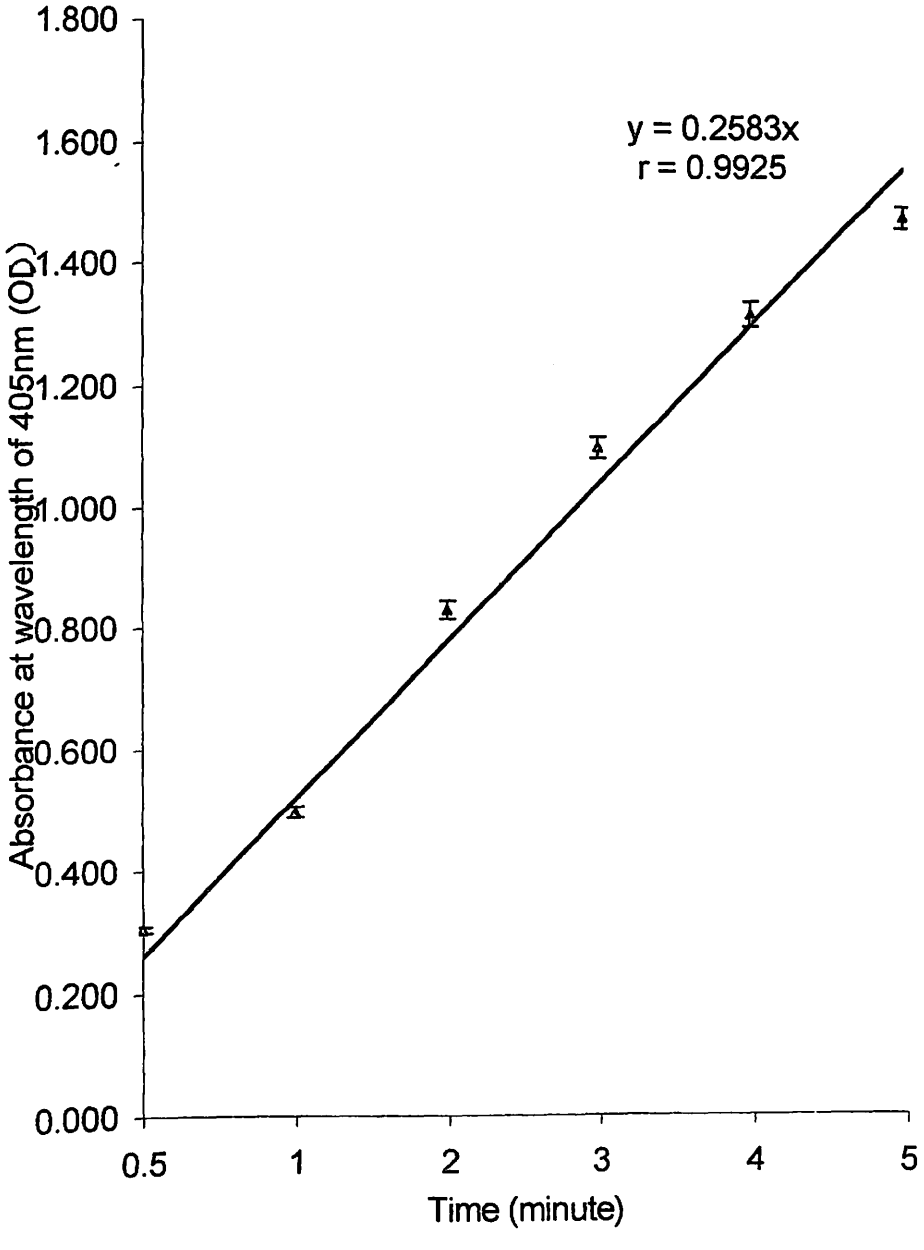


Figure 3.6: The absorbance of 60ku/ml purified tissue kallikrein activity against the time of 0 to 5 minutes.

3.5.2 Preparation of a standard graph for tissue kallikrein level

A standard graph for tissue kallikrein level using purified tissue kallikrein at different concentrations (from 0 to 10ku/ml) was prepared (Appendix G). This wide range of concentration was chosen since no data is available to quantify the tissue kallikrein level in fetoplacental tissue. Preparation of this standard graph was done as follows:

1. 2250µl of Tris HCl buffer pH 9.0, 0.05M was preincubated at 37°C in a sterile plastic tube.
2. After 5 minutes, 50µl of 0ku/ml purified tissue kallikrein was added, mixed well and incubated for 2 minutes at 37°C.
3. Then 200µl of S-2266 was added, mixed well and incubated. (Blank solution was also prepared by using the same reagents as above in the same order but substituting 200µl of Tris HCl buffer for the substrate S-2266 solution)
4. At four minutes, after adding S-2266, the absorbance for purified tissue kallikrein activity was measured using a spectrophotometer at a wavelength of 405nm.
5. These steps were repeated using different concentrations of purified tissue kallikrein (i.e. 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10ku/ml).
6. The results presented are the mean values of triple determinations. A standard graph was plotted with different concentrations of standard tissue kallikrein on the X-axis (ku/ml) and the absorbance on Y-axis (OD).

3.5.3 Determination of active tissue kallikrein level in each sample

Active tissue kallikrein level in each sample was determined as follows:

1. 2250µl of Tris HCl buffer pH 9.0, 0.05M was preincubated at 37°C in plastic sterile test tubes.
2. After 5 minutes, 50µl of each sample was added, mixed well and incubated for 2 minutes at 37°C.
3. Then 200µl of 2mM S-2266 was added and well mixed. (Blank solution was also prepared by using the same reagents as above in the same order but substituting 200µl of Tris HCl buffer for the substrate S-2266 solution)
4. At four minutes, after adding S-2266, the absorbance for tissue kallikrein activity in each sample was measured using a spectrophotometer at a wavelength of 405 nm.
5. The results presented are the mean values of double determinations. The active tissue kallikrein level in each sample was obtained from the standard graph and then calculated as kallikrein unit in 1mg fetoplacental tissue (ku/mg).

3.5.4 Determination of total tissue kallikrein level in each sample

Total tissue kallikrein level in each sample was determined by trypsinizing the sample to activate the inactive tissue kallikrein that might be present in the sample and followed by the steps in 3.5.3 as above. These were done as follows (Sharma *et.al*, 1998):

1. Trypsinized sample was prepared by adding 20µg of trypsin,TPCK (Sigma, USA; Appendix H) to 500µl of sample. This was well mixed and incubated at 37°C.
2. After 30 minutes, 100µg of SBTI (Sigma, USA) was added to stop the reaction.

3. Then 50μl of trypsinized sample was added to 2250μl of preincubated Tris HCl buffer pH 9.0, 0.05M at 37°C for 15 minutes. This again was mixed well and incubated again at 37°C for 2 minutes.
4. This was followed by the addition of 200μl of 2mM S-2266, which was mixed well and then incubated at 37°C. (Blank solution was also prepared by using the same reagents as above in the same order but substituting 500μl of Tris HCl, 50μl of trypsinized Tris HCl and 200μl of Tris HCl buffer for the sample, trypsinized sample and S-2266 solution, respectively)
5. At 4 minutes, after adding S-2266, the absorbance for total tissue kallikrein activity in each sample was measured using a spectrophotometer at a wavelength of 405nm.
6. The results presented are the mean values of double determinations. The total tissue kallikrein level in each sample was obtained from the standard graph and then calculated as kallikrein unit in 1mg fetoplacental tissue (ku/mg).

3.5.5 Calculation of inactive tissue kallikrein level in each sample

The difference between total and active tissue kallikrein was calculated as inactive tissue kallikrein level in each corresponding sample. The results were expressed as kallikrein unit in 1mg fetoplacental tissue (ku/mg).

3.6 Determination of the molecular weight of tissue kallikrein in each sample of the various fetoplacental tissues obtained from both groups using Western blot analysis

The molecular weight of tissue kallikrein was first determined in the samples of maternal placenta obtained from NP women. This was then followed by determination of tissue kallikrein in the samples of various fetoplacental tissues to see any differences between the two groups. The samples were prepared as steps in 3.3 except that the volume of KH solution added to each fetoplacental tissue was 1ml for a 400mg of tissue. The samples and standard protein (purified human urinary tissue kallikrein - Calbiochem®, Germany) were then separately loaded onto sodium dodecylsulfate (SDS) polyacrylamide gel followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using Mini-Protean II Cell (Bio-Rad, USA), to separate the proteins based on their molecular size. The separated proteins were then electrophoretically transferred to a nitrocellulose paper, using Mini-Transblot Electrophoretic Transfer Cell (Bio-Rad, USA). The paper was then incubated in a first antibody followed by a second antibody. The second antibody was then visualized by a precipitable substrate (Weerasinghe & Gadsby, 1992 & Hermann *et.al*, 1996 with some modifications, Figure 3.7).

3.6.1 Preparation of sodium dodecylsulfate (SDS)-polyacrylamide gel

The SDS-polyacrylamide gel was prepared as follows:

1. Glass plate sandwich, using 2 clean glass plates & 2 spacers, were prepared.

2. The sandwich was locked to the casting stand (leaking was checked with deionized water).
3. A separating gel solution (7.5%) was prepared in a flask (Appendix I) and transferred to the center of the sandwich.
4. A layer of deionized water (about 1cm thick) was pipetted slowly to cover the top of the resolving gel to make the upper level smooth. It was allowed to polymerize for 15 minutes at room temperature.
5. Then the layer of deionized water was poured off completely.
6. A stacking gel solution (4%) was prepared (Appendix J) in a flask.
7. The stacking gel solution was pipetted onto the resolving gel followed by the insertion of a Teflon comb (with 10 wells) into it.
8. Additional stacking gel solution was added to completely fill the spaces in the comb.
9. The solution was allowed to polymerize for 15 minutes at room temperature.

3.6.2 Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was done as follows:

1. 4 protein samples were prepared as follows:
    a) 10μl standard protein (human urinary tissue kallikrein – 3.1μg)
    b) 10μl sample (from maternal placenta - 4mg)
    c) 10μl standard protein and 10% 2-mercaptoethanol (Bio-Rad, USA)
    d) 10μl sample and 10% 2-mercaptoethanol

    Each protein sample was diluted separately in 10μl electrophoresis sample buffer (Appendix K) and 0.005g bromophenol blue (FisherChemical, USA).

(Sample and standard protein without 2-mercaptoethanol were also used in this study to see if any tissue kallikrein subunit might be present in each solution as described by Weerasinghe & Gadsby, 1992).

2. All samples were boil separately for 10 minutes at 100°C and then centrifuged at 120rpm for 5 minutes.
3. The Teflon comb was removed carefully & the bubbles were flushed with deionized water.
4. The sandwich was placed into the buffer chamber.
5. A liter of running buffer (Appendix L) was poured into the lower & upper chamber till the sample wells of the stacking gel were filled with the buffer.
6. 10μl aliquot of each protein sample was separately loaded with a Hamilton syringe into the bottom of each well. This was done in duplications.
7. Then 5μl molecular weight marker (Gibco-BRL, USA) was loaded into 2 most lateral wells.
8. The gel was run for 1 hour at 0.25Amp. of current.
9. The sandwich was removed from the chamber and one glass plate was opened carefully.
10. The stacking gel was removed.
11. The separating gel was then cut into two portions with one portion preserved for staining and the other for transblotting.
12. The staining of the gel was done with Cosmassie Blue solution (Appendix M) for 1 hour followed by destaining in a destaining solution (Appendix N) for subsequent photograph and kept as a permanent record.

3.6.3 Transblotting to nitrocellulose membrane

1. The other portion of gel (as prepared in 3.6.2) was placed into a container filled with running buffer.
2. Prewetted (i.e. with running buffer) Scotch-Brite pad was put on the plastic support (black for cathode).
3. A Whatman filter paper was put under the gel, which was in the plastic container filled with running buffer. Then the gel, with Whatman filter paper under it, was placed onto the previously prewetted Scotch-Brite pad.
4. A prewetted (i.e. with running buffer) nitrocellulose membrane was placed directly on the gel. All air bubbles were removed with a glass rod.
5. Another prewetted Whatman filter paper was placed on the nitrocellulose membrane and all air bubbles were again removed.
6. Then, another prewetted Scotch-Brite pad was placed on top of this Whatman filter paper to support both gel and nitrocellulose membrane.
7. The plastic support was closed and placed into the electroblotting apparatus in the correct orientation (black for cathode & white for anode).
8. The tank was filled with transferring buffer (Appendix O).
9. The proteins in the gel were transferred at 0.25Amp. of current for 1 hour at 4°C.
10. After complete transfer, the nitrocellulose membrane was placed in Ponceau S solution (Appendix P) for 5 minutes to stain the proteins.
11. Then it was destained in deionized water for a few times, the molecular weight of the standard marker band was marked and the marker lane was cut.

12. The other portion of nitrocellulose membrane was then placed in blocking solution (Appendix Q) to block non-specific antibody sites on the membrane for 1 hour.
13. Then it was washed with deionized water for 3 times.
14. The membrane was then incubated in 1:2000 dilution of rabbit anti-human urinary tissue kallikrein (as primary antibody; Calbiochem®, Germany) in phosphate buffer saline (PBS) solution (Appendix R) for overnight.
15. Then it was washed with Tween-20 solution (Appendix S) 6 times every 10 minutes.
16. This was followed by incubation with 1:5000 dilution of Horseradish peroxidase-conjugated goat anti-rabbit Ig-G (as secondary antibody; Calbiochem®, Germany) in PBS solution for 1 hour and was covered with aluminium foil.
17. Then, it was washed with Tween-20 solution for 6 times every 10 minutes.
18. The membrane was then incubated in 4-chloro-naphtol substrate solution (Appendix T) for 15 minutes and covered with aluminium foil.
19. The staining reaction was then stopped by washing with deionized water twice. The stained membrane was photographed and kept as a permanent record.

The molecular weight of tissue kallikrein in each sample (i.e. amnion, chorion laeve, placental plate chorion, fetal placenta and maternal placenta) obtained from both groups was determined using the same steps as in 3.6.2 and 3.6.3. All samples were prepared in the presence of 2-mercaptoethanol in order to determine the molecular weight of tissue kallikrein in each sample.